Development of Spontaneous Airway Changes Consistent with Human Asthma in Mice Lacking T-bet

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Human asthma is associated with airway infiltration by T helper 2 (T_H2) lymphocytes. We observed reduced expression of the T_H1 transcription factor, T-bet, in T cells from airways of patients with asthma compared with that in T cells from airways of nonasthmatic patients, suggesting that loss of T-bet might be associated with asthma. Mice with a targeted deletion of the T-bet gene and severe combined immunodeficient mice receiving $CD4^+$ cells from T-bet knockout mice spontaneously demonstrated multiple physiological and inflammatory features characteristic of asthma. Thus, T-bet deficiency, in the absence of allergen exposure, induces a murine phenotype reminiscent of both acute and chronic human asthma.

Human asthma is associated with reversible airway obstruction, airway inflammation, airway hyperresponsiveness (AHR) and, airway remodeling. Murine models of asthma mimic many of the features of the human disease. In these models, the production of various cytokines including interleukin-4 (IL-4), IL-5, and IL-13 have been associated with the development of an asthma-like phenotype (*1*). In an adoptive-transfer model, enhanced expression of interferon-γ (IFN-γ) by T_H1 cells in the airway protects against allergic disease, but the presence of T_H1 cells does not attenuate T_H2 cell–induced airway hyperreactivity and inflammation (*2–6*). T-bet, a T_H1-specific T-box transcription factor, transactivates the IFN-γ gene in T_H1 cells and has the unique ability to redirect fully polarized T_H2 cells into T_H1 cells, as demonstrated by simultaneous induction of IFN-γ and repression of IL-4 and IL-5 (*7–10*).

We had previously observed that T-bet transcripts could be detected in lung tissue. Therefore, we investigated whether any differences were apparent in the levels of T-bet expressed in the lungs of patients with allergic asthma compared with T-bet levels in nonasthmatic patients. Immunohistochemistry with a monoclonal antibody (mAb) to T-bet (*11*) revealed expression of T-bet in nonasthmatic control lungs with significantly less expression in seven patients with allergic asthma (Fig. 1B). Double staining for CD4 and T-bet in consecutive sections showed that most of the cells expressing T-bet were $CD4^+$ T cells. These observations in human tissue led us to speculate that T-bet deficiency might recapitulate aspects of the asthmatic phenotype. To test this hypothesis directly, we examined T-bet–deficient mice for changes associated with human asthma.

In the absence of immunologic sensitization and challenge, mice either heterozygous ($T\text{-bet}^{+/-}$) or homozygous ($T\text{-bet}^{-/-}$) for a targeted deletion of T-bet, compared with wild-type (WT) mice, exhibited AHR to methacholine, as measured by the noninvasive enhanced pause (Penh) method (*12*) (Fig. 2A). These findings were confirmed in another cohort of mice in which airway responsiveness was measured by invasive techniques (*13*) after intravenous administration of methacholine (*14*). Histopathologic analysis of the airways of $T\text{-bet}^{-/-}$ mice at base-

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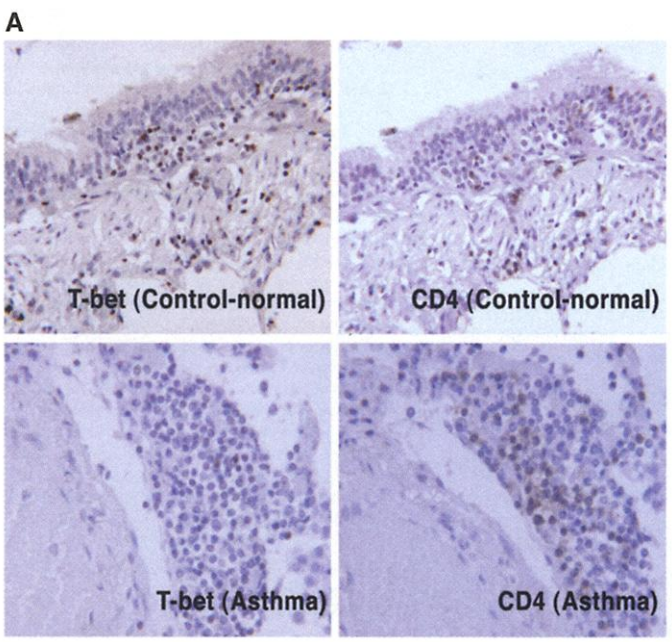

Fig. 1. T-bet expression is down-regulated in human asthmatic airways. (**A**) (Left) Comparison of immunohistochemical expression and localization of T-bet in the human airways of control subjects (top) and asthmatic subjects (bottom) after staining with a mAb directed against T-bet (10 μg/ml). (Right) Consecutive sections were immunostained for CD4. Negative controls were performed by omitting the first antibody. Comparison between the upper and lower panels illustrates the decrease of T-bet expression in asthmatic airways. Even though numerous $CD4^+$ T cells are present peribronchially, these cells are T-bet–negative. Original magnification, ×200. (**B**) Enumeration of T-bet (left) and $CD4^+$ cells (right) in two control groups and in patients with asthma. $*P = 0.05$, $**P = 0.00050$.

line demonstrated peribronchial and perivenular infiltration with eosinophils and lymphocytes as compared with control WT littermates (Fig. 2B) (*14*). T-bet$^{+/-}$ heterozygous mice, which display only a 50% reduction of T-bet protein expression, displayed a phenotype very similar to that of mice with a complete absence of T-bet (*15*).

Airway remodeling, a feature of chronic human asthma characterized by deposition of collagen III below the basement membrane, was present in naïve T-bet–deficient mice (Fig. 2C). The thickness of this subepithelial collagen layer was evaluated in WT and in T-bet–deficient (T-bet$^{-/-}$ and T-bet$^{+/-}$) animals (Fig. 2D). In the WT mice, there was minimal deposition of collagen beneath the basement membrane, whereas in the T-bet–deficient mice the subbasement membrane collagen layer was significantly thicker than it was in WT mice. In addition to a thickened collagen layer, there were increased numbers of bronchial myofibroblasts in the T-bet–deficient mice (Fig. 2E). These data indicate that the airways of T-bet–deficient mice undergo remodeling similar to that observed in humans with chronic asthma (*16*).

Transforming growth factor–β1 (TGF-β1, a potent stimulator of tissue fibrosis), tumor necrosis factor–α (TNF-α), IL-4, and IL-13 were recovered in increased amounts from the bronchial alveolar lavage fluid (BALF) of T-bet$^{-/-}$ mice compared with that from WT mice (*14*, *17*). T-bet deficiency induced a selective alteration in patterns of cytokine expression in BALF because no significant changes were observed in IL-10 and IL-6 production (*15*).

Although antigen sensitization and challenge result in an induction of AHR in WT mice, animals with a targeted deletion of T-bet did not develop further AHR after similar treatment (*14*). However, mice with a targeted deletion of T-bet were more responsive without allergen challenge than were WT mice following antigen challenge. Antigen sensitization and challenge induced increased levels of IL-5 in WT mice, but among the T-bet–deficient animals, the levels of IL-5 were high before challenge and did not increase following challenge (*14*).

Adoptive transfer of splenic CD4$^+$ cells (or saline in control mice) from different groups of ovalbumin (OVA)-sensitized mice into histocompatible severe combined immunodeficient (SCID) mice was used to examine the importance of these cells in mediating the asthmatic phenotype. To enhance the localization of the transferred T cells into the lungs of mice, we administered OVA aerosol 1 day before and 3 days after adoptive transfer. Four days after cell transfer, recipients of WT spleen CD4$^+$ cells exhibited airway responsiveness comparable to that of WT mice that received OVA sensitization and challenge. Mice that had been reconstituted with CD4$^+$ cells lacking T-bet showed increased AHR as compared with mice reconstituted with CD4$^+$ cells derived from WT littermates (Fig. 3A) and similar to that of OVA-sensitized mice lacking T-bet. CD4 staining of BALF cells harvested after measurement of lung mechanics was performed to ensure that CD4 cells were recruited to the lung; the pro-

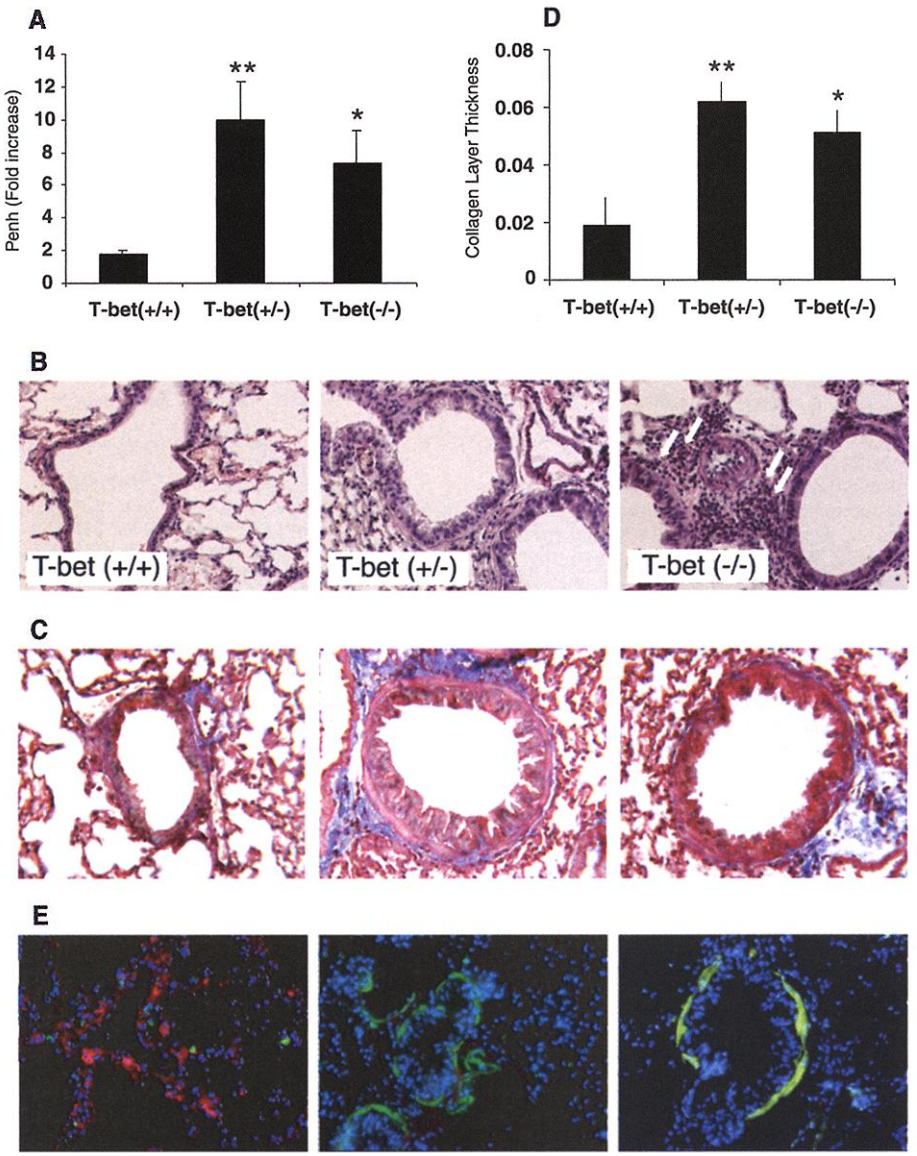

Fig. 2. Loss of T-bet results in increased AHR. (**A**) AHR was measured in 4- to 6-week-old WT and T-bet–deficient mice (*20*) by measuring Penh (enhanced pause) with a noninvasive whole-body plethysmograph system (Buxco Electronics, Sharon, CT) before and after administration of methacholine aerosol (200 mg/ml, Sigma; Ultrasonic 5500D nebulizer, Devilbliss, Somerset, PA). Penh is a parameter derived from analysis of the respiratory pattern; increases in Penh are related to an increase in pulmonary resistance (*12*). Results are reported as maximal fold increase of Penh relative to baseline values. Data represent the mean ± SEM of at least eight mice per group (*$P <$ 0.05, **$P < 0.01$ for T-bet–deficient mice compared with WT mice; Student's two-tailed t test for independent events). (**B**) Histological features of naïve WT and T-bet–deficient mice. Lungs from WT and knockout T-bet$^{+/-}$ and T-bet$^{-/-}$ mice were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Airway inflammation is shown by an increased presence of peribronchial and perivascular infiltration with eosinophils and lymphocytes in T-bet$^{-/-}$ mice. Original magnification, ×200. (**C**) Airway remodeling in T-bet$^{-/-}$ mice. Increased collagen deposition is apparent around the airways of T-bet$^{-/-}$ mice compared with that around the airways of WT mice (Trichrome/Masson stain). Original magnification, ×200. (**D**) Peribronchial collagen was quantified by measuring the collagen thickness (μm) beneath the bronchial epithelium normalized by dividing by the bronchial diameter (μm). There was a significantly greater amount of collagen around the airways of T-bet–deficient mice compared with that around the airways of WT mice (*$P < 0.05$, **$P < 0.01$). (**E**) Increased immunostaining of myofibroblasts in the airways of T-bet–deficient mice compared with that in the airways of WT mice. Staining for T-bet (red) and alpha smooth muscle actin (green) is shown (*14*).

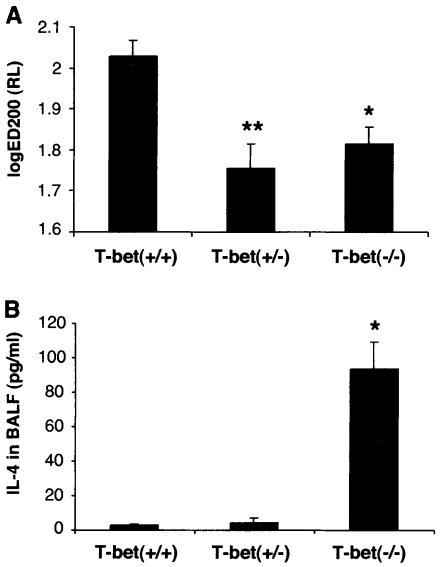

Fig. 3. Adoptive transfer of spleen CD4-OVA–specific T cells derived from T-bet–deficient mice induced increased AHR and increased IL-4 production in the airways. After OVA sensitization, CD4-positive cells were sorted from the spleen of WT or T-bet $^{-/-}$ mice by using anti-CD4 beads (Dynal, NY) (1.5×10^6 cells/mouse) and transferred intraperitoneally into histocompatible SCID mice (C.B.-17Icr $^{scid/scid}$; Taconic laboratories, Germantown, PA). **(A)** AHR in response to an increasing concentration of intravenous methacholine was determined by standard measures of pulmonary mechanics in anesthetized and mechanically ventilated mice. The effective dose of intravenous methacholine required to double pulmonary resistance is termed ED_{200} R_L; higher values of ED_{200} R_L denote lower levels of airway responsiveness (*12*). Adoptive transfer of OVA-specific CD4 cells derived from mice lacking T-bet induced AHR in SCID mice as compared with similar mice reconstituted with OVA-specific CD4 cells derived from WT matched littermates (*P = 0.05, **P = 0.0029). **(B)** An increase in IL-4 production in the BALF was observed only in SCID mice reconstituted with CD4 cells derived from T-bet$^{-/-}$) mice (*$P < 0.05$).

portion of lymphocytes that were CD4$^+$ was 38.9 ± 2.2% (WT$^{+/+}$ mice), 39.57 ± 6.48% (CD4 T-bet$^{+/-}$ mice), and 38.5 ± 5.48% (T-bet$^{-/-}$ mice). In addition, the lungs of SCID mice reconstituted with CD4$^+$ cells derived from T-bet$^{-/-}$ mice exhibited increased IL-4 in the BALF as compared with recipient mice reconstituted with spleen CD4$^+$ cells derived from WT mice (Fig. 3B), demonstrating that the AHR observed in T-bet$^{-/-}$ mice is T cell–mediated.

Our data demonstrate that targeted deletion of T-bet, in the absence of an induced inflammatory response, results in a physiological and inflammatory phenotype in murine airways similar to that created by allergen exposure in sensitized mice. T-bet–deficient mice demonstrate airway remodeling consistent with asthma that is reminiscent of the human disease. This phenotype exists in naïve mice spontaneously and is similar to that observed in mice following allergen sensitization and challenge. These data suggest that T-bet might be an attractive target for the development of anti-asthmatic drugs; the presence of the asthmatic phenotype in mice carrying only one copy of T-bet implies that subtle changes in T-bet expression or activity may provide a means of modulating the asthmatic response. In that regard, it is intriguing that in the mouse, T-bet is located on chromosome 11 in a region that has been identified as genetically linked to AHR in mice and recently reported as linked to asthma in humans.

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11. Paraffin sections from seven patients with chronic asthma were associated with histological signs of airway remodeling (collagen deposition below the basement membrane and hypertropy of the smooth muscle cells) and chronic airway inflammation (airway infiltration with eosinophils); sections from 13 histologically normal lung samples were taken at a distance from a region of lung containing nonasthmatic or allergic pathology. For immunohistochemical staining, lung sections were deparaffinized, pretreated in a microwave oven in EDTA-buffer (1 mM EDTA-NaOH, pH 8.0) for antigen retrieval, followed by incubation with 2% normal horse serum and either T-bet mAb (4B10) (1:100) or a human CD4 mAb (1:100) (Novovastra, UK). Specific binding was detected by standard methods (*18*).
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17. BALF and blood were collected and processed as described (*19*). Supernatants were analyzed by specific enzyme-linked immunosorbent assay for content of IFN-γ, IL-6, TNF-α, TGF-β, and IL-4 (Pharmingen, San Diego, CA) or IL-5 and IL-13 detection (R&D Systems).
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Distinct Effects of T-bet in T_H1 Lineage Commitment and IFN-γ Production in CD4 and CD8 T Cells

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T-bet is a member of the T-box family of transcription factors that appears to regulate lineage commitment in CD4 T helper (T_H) lymphocytes in part by activating the hallmark T_H1 cytokine, interferon-γ (IFN-γ). IFN-γ is also produced by natural killer (NK) cells and most prominently by CD8 cytotoxic T cells, and is vital for the control of microbial pathogens. Although T-bet is expressed in all these cell types, it is required for control of IFN-γ production in CD4 and NK cells, but not in CD8 cells. This difference is also apparent in the function of these cell subsets. Thus, the regulation of a single cytokine, IFN-γ, is controlled by distinct transcriptional mechanisms within the T cell lineage.

IFN-γ, a pleiotropic cytokine produced principally by CD4 T_H1 cells, CD8 T cells, and NK cells, is essential for both innate and adaptive immunity. It acts by binding to the IFN-γ receptor expressed on nearly all cell types (*1*, *2*) that is coupled to the Jak-STAT signaling pathway (*2*, *3*). Mice lacking IFN-γ, the IFN-γ receptor, or Stat1 display a profound disruption of both innate and adaptive immunity, resulting in death from infection by microbial pathogens and viruses (*4*–*7*). Humans with inactivating mutations in components of the IFN-γ signaling die at an early age from uncontrolled mycobacterial